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Neuregulin (heregulin) and betacellulin are two members of the epidermal growth factor (EGF) family of peptide growth factors. The receptors for these ligands are the erbB family of receptor protein tyrosine kinases. They include the EGF receptor, neu/erbB-2/HER2, erbB-3/HER3, and erbB-4/HER4. Not only have neuregulin and betacellulin been implicated in mammary carcinogenesis, but deregulated signaling by the erbB family receptors are also thought to play a causative role in these processes. However, the interactions of neuregulin and betacellulin with the erbB family receptors have not been thoroughly characterized.

Therefore, we have expressed the four erbB family receptors, singly and in pairwise combinations, in the Ba/F3 mouse pro-B-lymphocyte cell line. By stimulating the resulting panel of cell lines with neuregulin, betacellulin or EGF and assaying receptor tyrosine phosphorylation, we have identified which erbB family receptors are activated by these ligands. Furthermore, we have assessed ligand-induced coupling of receptors to cellular signaling pathways by measuring ligand stimulation of interleukin-3-independent growth in these cell lines. These data demonstrate that EGF, betacellulin, and neuregulin stimulate distinct patterns of erbB family receptor tyrosine phosphorylation and coupling to cellular signaling pathways. These differences may account for their different biological activities.

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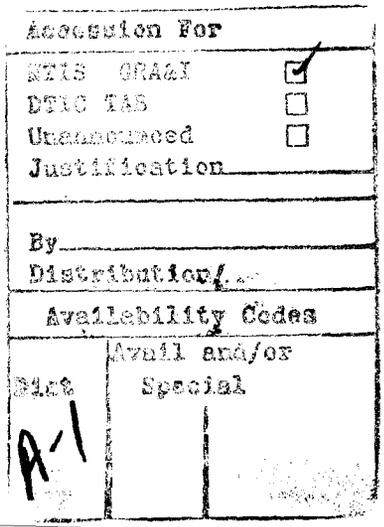

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Introduction

Introduction to the problem

Breast cancer is one of the leading causes of cancer death in women in the United States, with approximately 46,000 American expected to die of the disease in 1993 [Boring, *et al.*, 1993]. One model is that breast cancer results from disruptions in the normal hormonal regulation of mammary gland epithelial cell proliferation and differentiation. We have undertaken experiments to characterize the regulation of signaling by the epidermal growth factor receptor (EGFR) family of protein tyrosine kinases by their ligands, the epidermal growth factor (EGF) family of peptide hormones. We also have begun experiments that assess the effects of this signaling on the proliferation, differentiation, and malignant growth transformation of mammary epithelial cells Therefore, these experiments are beginning to shed light on the role that these proteins play in breast carcinogenesis.

The EGF family/erbB receptor family signaling network

Deregulated signaling by the four receptor tyrosine kinases encoded by the erbB gene family (erbB-1/epidermal growth factor receptor [EGFR], neu/erbB-2/HER2 , erbB-3/HER3 , and erbB-4/HER4), has been implicated in a number of human cancers, including mammary cancer, ovarian cancer, gastric cancer, and glioblastoma [Reviewed in Hynes and Stern, 1994]. Understanding the normal and pathological functions of these receptors requires that their regulation by hormones be elucidated. However, there are at least 15 different agonists for erbB family receptors, including epidermal growth factor (EGF), transforming growth factor alpha (TGFα), amphiregulin (AR), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), cripto, epiregulin, and the several differentially spliced

variants of the neuregulins (NRGs), also known as gp30, heregulins, neu differentiation factors, glial growth factors, and acetylcholine receptor inducing activity [Reviewed in Groenen, *et al.*, 1994]. Furthermore, not only do some of these factors bind to and activate signaling by more than one receptor, but some of these ligands stimulate non-additive receptor interactions in cells expressing multiple erbB receptor family members [Reviewed in Earp, *et al.*, 1995]. For example, EGF activates neu when coexpressed with the EGFR, but EGF does not bind or activate neu expressed on its own [Akiyama, *et al.*, 1988; King, *et al.*, 1988; Stern and Kamps, 1988; Connelly and Stern, 1990]. This "transmodulation" activation of neu by EGFR apparently works through the formation of EGF-driven receptor heterodimers and receptor cross-phosphorylation [Goldman, *et al.*, 1990; Wada, *et al.*, 1990; Qian, *et al.*, 1992; Spivak-Kroizman, *et al.*, 1992].

Betacellulin (BTC) was initially identified as a factor in the conditioned medium of a mouse pancreatic β cell carcinoma (insulinoma) cell line that was mitogenic for Balb/C 3T3 cells. The 80 amino acid mature BTC protein is derived from a 177 amino acid membrane-bound precursor, contains the six conserved cysteine residues that are arranged in a characteristic pattern common to all of the members of the EGF family, and has significant overall homology to mature EGF, TGF-α, AR, HB-EGF, and NRGs [Sasada, *et al.*, 1993; Shing, *et al.*, 1993; Reviewed in Groenen, *et al.*, 1994]. Binding of human recombinant BTC to the A431 human adenocarcinoma cell line and the MDA-MB-453 human breast carcinoma cell line, both of which overexpress EGFR, can be competed with an excess of EGF, suggesting that BTC is a ligand for the EGFR [Watanabe, *et al.*, 1994]. However, previous studies have not assessed BTC binding to other erbB family receptors or BTC stimulation of signaling by erbB family receptors. Little is known about BTC function *in vivo*. While its expression in the BTC-3 mouse insulinoma cell line

[Shing, *et al.*, 1993] and the MCF-7 human breast adenocarcinoma cell line [Sasada, *et al.*, 1994] implies that BTC regulates the proliferation of pancreatic and breast cells and may play a causative role in breast and pancreatic cancer, the receptor(s) for BTC must be identified before definitive studies of the physiologic effects of BTC can be undertaken.

NRGs were initially identified as candidate neu ligands by their ability to induce neu tyrosine phosphorylation. The longest forms of NRG contain several different modular domains, including a kringle fold, a C-2 immunoglobulin-like domain, a putative heparan sulfate proteoglycan attachment site, sites for N- and O-linked glycosylation, an EGF homology domain, a hydrophobic membrane-spanning domain, and an intracellular domain of variable length [Holmes, *et al.*, 1992; Peles, *et al.*, 1992; Wen, *et al.*, 1992; Marchionni, *et al.*, 1993; Corfas, *et al.*, 1995]. Tissue-specific alternative splicing of NRG transcripts from a single gene results in many NRG isoforms containing different sets of these motifs. Moreover, alternative splicing also produces two types of EGF domain, denoted α and β [Wen, *et al.*, 1994]. α and β isoforms have different biological activities, which may in part reflect their differential binding affinities to cells expressing receptors for NRG [Lu, *et al.*, 1995].

NRGs are likely to play a significant role in regulating cellular proliferation and differentiation *in vivo*. NRGs were initially purified from medium conditioned by *ras*-transformed Rat-1 fibroblasts [Peles, *et al.*, 1992] or by the MDA-MB-231 human mammary tumor cell line [Holmes, *et al.*, 1992], suggesting that NRGs establish or maintain the growth-transformed phenotype. NRG also affects the proliferation and differentiation of cultured mammary cells. NRG stimulates [Holmes, *et al.*, 1992] or inhibits [Peles, *et al.*, 1992; Wen, *et al.*, 1992] the *in vitro* proliferation of human mammary tumor cells, which frequently overexpress erbB

family receptors [Reviewed in Hynes and Stern, 1994], while NRG stimulates proliferation and milk protein synthesis in a cultured mouse mammary epithelial cell line [Marte, *et al.*, 1995]. NRG may also promote wound healing. A single NRG isoform accelerates epidermal migration via increased terminal differentiation of epidermal cells and stimulates integrin expression in the epidermis during wound healing, while wounding stimulates NRG expression in dermal fibroblasts adjacent to the wound [Danilenko, *et al.*, 1995]. NRG also modulates the differentiation and proliferation of neuroectodermal cells. NRGs act as glial cell growth factors [Marchionni, *et al.*, 1993], may specify a glial cell fate for neural crest stem cells [Shah, *et al.*, 1994], appear to mediate axon-induced mitogenesis of Schwann cells [Orr-Utreger, *et al.*, 1993] and stimulate acetylcholine receptor synthesis at neuromuscular junctions [Corfas, *et al.*, 1993; Falls, *et al.*, 1993; Jo, *et al.*, 1995]. Furthermore, NRG expression patterns suggest important functions in neurogenesis and in mesenchymal/epithelial interactions during development [Marchionni, et al., 1993; Orr-Utreger, *et al.*, 1993; Meyer, et al., 1994; Corfas, et al., 1995].

The physiological responses to agonists for erbB family receptors depends on their ability to coordinately activate multiple receptors that are differentially expressed and have different signaling capabilities. Although NRGs were initially purified by their ability to induce neu tyrosine phosphorylation, and were thought to be ligands for neu, NRG does not bind neu and/or induce neu tyrosine phosphorylation in a variety of cell types or in solution [Culouscou, *et al.*, 1993; Peles, *et al.*, 1993; Plowman, *et al.*, 1993b; Sliwkowski, *et al.*, 1994; Tzahar, *et al.*, 1994]. Instead, NRG binds erbB-3 [Carraway, *et al.*, 1994; Kita, *et al.*, 1994; Sliwkowski, *et al.*, 1994; Tzahar, *et al.*, 1994] and erbB-4 [Plowman, *et al.*, 1993a; Plowman, *et al.*, 1993b; Culouscou, *et al.*, 1994]. Co-expression of erbB-3 or erbB-4 with neu permits NRG-induced tyrosine phosphorylation of neu, presumably through the formation of

neu/erbB-3 or neu/erbB-4 heterodimers [Plowman, *et al.*, 1993b; Carraway, *et al.*, 1994; Kita, *et al.*, 1994; Sliwkowski, *et al.*, 1994]. Despite the many combinatorial possibilities afforded by assortment of four different receptors, interactions of erbB family members with their agonists have only been investigated in a piecemeal fashion. Receptors of different species origins have been mixed in gene transfer experiments, only a subset of receptor combinations have been tested, and interpretation is hampered by the variety of cell backgrounds used and in many cases by the failure to determine the endogenous erbB family receptor expression in the cell lines used. Finally, the hormone-regulated coupling of different erbB family receptors and combinations of receptors to different downstream signaling pathways has not been systematically investigated for any EGF family agonist, including NRGs and BTC.

Experimental approach

In order to address these issues we have undertaken a parallel analysis of the aggregate signaling potential of the erbB receptor family. We have expressed all four human erbB family receptors, singly and in each pairwise combination, in the interleukin-3-dependent mouse Ba/F3 pro-B-lymphocyte cell line. By assessing erbB receptor tyrosine phosphorylation in the resulting panel of cell lines following stimulation with EGF, NRG-β, or BTC, we have performed the first comprehensive evaluation of ligand-induced erbB family receptor activation. Furthermore, by assessing the induction of IL-3-independent growth in this panel of cell lines following stimulation with EGF, NRG-β, or BTC, we have also evaluated ligand-induced erbB family receptor coupling to cellular signaling pathways. These data establish that EGF, NRG-β, and BTC exhibit distinct patterns of erbB receptor activation and coupling to cellular signaling pathways. Moreover, these data

suggest several distinct mechanisms by which biological responses are specified by interactions among erbB family receptors and their agonists.

Materials and Methods

Neuregulin-β.

Neuregulin-β (NRG-β) has been supplied on a collaborative basis by James D. Moyer, Brad C. Guarino, and Glenn C. Andrews, Pfizer Central Research, Groton, CT. NRG-β has been supplied as a refolded, chemically synthesized 65-mer peptide [Barbacci, *et al.*, 1995] corresponding to amino acids 177 to 241 of the NRG β1 isoform (amino acid residues are numbered according to [Holmes, *et al.*, 1992]).

Betacellulin.

Human recombinant betacellulin has been supplied on a collaborative basis by Sharon Buckley and Gregory D. Plowman, Sugen, Inc., Redwood City, CA. To facilitate refolding and purification, human recombinant betacellulin was produced as an epitope-tagged fusion with human amphiregulin. The pPL-Lambda (Pharmacia) thermoinducible bacterial expression vector was modified to remove the EcoRI, BamHI, and SmaI sites upstream from the PL promoter and a human betacellulin transcription unit was inserted into the unique HpaI site within the N gene. The insertion contained the lac and Cro gene Shine-Delgarno ribosome binding sites; a unique BglII cloning site; an initiating methionine codon; the nucleotide sequence encoding 34 amino acids of the human amphiregulin precursor (Val107 - Arg140), the 50 amino acid EGF-structural motif of human betacellulin (Arg31 - Tyr80), and a 9 amino acid hemagglutinin epitope sequence (PYDVPDYAS); a stop codon; unique EcoRV and XbaI restriction sites; and transcription termination sequences. The resulting plasmid, pPLABTC-Tag, was transformed into competent E. Coli N4830-1 and grown at 30°C in 1 liter LB media with 50 ug/ml ampicillin to an Abs_{600} of 0.7. Cultures were then induced by incubation at 42°C for 18-24 hr.

Following induction, cells were harvested by centrifugation at 5000xg, washed in STE buffer (50 mM Tris, pH 8.0/200 mM NaCl/2 mM EDTA). The pellet was resuspended in STE containing 2 mM 2-mercaptoethanol, and lysed by addition of 0.2 mg/ml lysozyme followed by addition of Triton X-100 and Zwittergent (CalBiochem) to 1%. To ensure lysis and solubilization of non-inclusion body protein, the preparation was sonicated for 2 min, and centrifuged at 13000xg for 10 min. The pellet was resuspended in STE and sonicated again for an additional 1 min. The slurry was then layered on a 40% sucrose cushion and centrifuged at 13000xg for 10 minutes at 4°C. The inclusion body pellet was resuspended in 6 M guanidine-HCl (GuHCL)/50 mM CAPS, pH 11.0.

The Betacellulin inclusion body preparation was diluted to 60 mM GuHCl with 50 mM CAPS, pH 11.0/1 mM EDTA/1.25 mM reducing glutathione/0.5 mM oxidizing glutathione. The final protein concentration was 50-100 mg/ml by Biorad protein assay. Refolding was achieved by incubation at 4°C for 18-24 hr. The solution was then dialyzed against 50 mM sodium phosphate (NaP), pH 7.5, and successively filtered through 5 mm, 0.45 mm, and 0.22 mm filters or subjected to 60,000xg centrifugation prior to cation exchange chromatography. Alternatively, the refolded material was buffer exchanged by ultrafiltration against 3 volumes of 50 mM NaP, pH 7.5.

Cleared, refolded bacterially-produced betacellulin was loaded on a cation exchange column (Bakerbond CSx) equilibrated with 40 mM NaP pH 7.0. The flow rate was 1.25 ml/min and the chromatography was carried out at room temperature. The column was washed with 20 column volumes of 40 mM NaP, pH 7.0, or until a stable baseline was achieved. The betacellulin was eluted with a 50 ml linear gradient of 0.2 -1 M NaCl in 40 mM NaP, pH 7.0. The peak fractions were at ~550 mM NaCl as determined by reactivity in a hemagluttinin ELISA. The peak fractions

from cation exchange chromatography were pooled, diluted to 0.2 M NaCl with 40 mM NaP, pH 7.0, and applied to an FPLC TSK-heparin 5PW column (TosoHaas). The flow rate was 1 ml/min. The column was then washed with 40 mM NaP, pH 7.0 and bound protein was eluted with a 30 ml linear gradient of 0 - 1.0 M NaCl in 40 mM NaP, pH 7.0. The recombinant tagged betacelluin eluted at ~800 mM NaCl, and migrated as a single Coomassie stained band on 15% SDS-PAGE.
Betacellulin activity was measured using an EGFR tyrosine phosphorylation assay (Thorne and Plowman, 1994). Recombinant betacellulin produced as a tagged fusion construct in bacteria had an activity indistinguishable from that produced transiently in COS cells using an expression construct containing the full length human betacellulin sequence.

Cell lines and cell culture.

The Ba/F3 mouse pro-B-lymphocyte cell line [Palacios and Steinmetz, 1985] and its derivatives were grown in RPMI (Gibco/BRL) supplemented with 10% fetal calf serum (Sigma) and Interleukin-3 (IL-3) supplied as 10% conditioned medium from the WEHI-3B mouse myelomonocytic leukemia cell line [Daley and Baltimore, 1988]. Ba/F3 derivatives transformed with constructs expressing erbB family receptors were grown in medium supplemented with 200ug/ml G418 (Gibco/BRL).

Plasmid constructions.

The SacII-XhoI fragment of pCO12EGFR [Velu, *et al.*, 1987], which contains the full-length human EGFR cDNA, was subcloned into the SmaI site of pBluescript SK-, generating pSKEGFR. The EGFR expression vector pLXSN-EGFR used in the experiments described here was constructed by cloning the 4.2Kb XhoI fragment of pSKEGFR, which contains the complete human EGFR cDNA, into the XhoI site of

the recombinant retroviral expression vector plasmid pLXSN, which carries a neomycin resistance gene under the transcriptional control of the SV40 early promoter [Miller and Rosman, 1989]. The neu expression vector pLXSN-Long-Neu was constructed by cloning the 4.8Kb NruI to DraI fragment of pCDNEU [Plowman, et al., 1993b], which contains the complete human neu cDNA as well as 714bp of vector sequences 5' to the neu transcriptional start site, into the HpaI site of pLXSN. Subsequently, the vector sequences 5' to the neu transcriptional start site were removed by cloning a 4.1Kb XhoI fragment of pLXSN-Long-Neu into the XhoI site of pLXSN, generating the neu expression vector pLXSN-Neu used in these studies. The erbB-3 expression vector pLXSN-erbB-3 was constructed by cloning the 4.3Kb BssHII fragment of pBSHER3X [Plowman, *et al.*, 1990], which contains the complete human erbB-3 cDNA, into the HpaI site of pLXSN. The erbB-4 expression vector pLXSN-erbB-4 was constructed by cloning the 4.6Kb SnaBI to SmaI fragment of pCH4M2 [Plowman, *et al.*, 1993a], which contains the complete human erbB-4 cDNA, into the HpaI site of pLXSN.

Generation of recombinant Ba/F3 derivatives.

10 ug of a single expression vector directing the expression of an erbB family receptor or 5 ug each of a pair of expression vectors were linearized by digestion with restriction endonucleases and ligated to form concatamers. These were electroporated into $2x10^7$ Ba/F3 cells in 0.5 ml Tris-buffered saline using a 0.4cm gap cuvette and a BioRad Gene Pulser set at 200V and 960μF. Cells were immediately diluted into 50 ml culture medium and were incubated for 48 hours at 37°C, then were seeded in 96-well dishes at $5x10^4$ cells per well in medium supplemented with 400μg/ml G418. Drug-resistant lines were expanded and screened for expression of the appropriate erbB family receptor(s). Positive lines were subcloned by limiting

dilution and rescreened for receptor expression to ensure homogeneity. The cell lines characterized here are named as follows: LXSN/1 (vector control); EGFR/3; neu/5 and neu/12C; erbB-3/3; erbB-4/7; EGFR + neu/5D; EGFR + erbB-3/4A; EGFR + erbB-4/2A; neu + erbB-3/7A; neu + erbB-4/15A; and erbB-3 + erbB-4/2B.

Stimulation and analysis of erbB family receptor tyrosine phosphorylation.

$4x10^8$ recombinant Ba/F3 cells were washed in phosphate-buffered saline (PBS), and resuspended in 50ml RPMI supplemented with IL-3. The cells were incubated for 6 hours at 37°C, washed in PBS, and resuspended in 2-3ml PBS. Remaining steps were performed cold or on ice. The cells were transferred in three to five 0.5-1.0 ml portions to microcentrifuge tubes. NRG-β, betacellulin, human recombinant EGF (Collaborative Biomedical) or the anti-neu agonistic monoclonal antibody TAb 250 [Langton, *et al.*, 1991; Shawver, *et al.*, 1994] was added at a final concentration of 94ng/ml (NRG-β), 150ng/ml (betacellulin), 100ng/ml (EGF), or 10ug/ml (TAb 250). Control samples remained untreated or were treated with PBS. Following a 10-minute incubation, cells were pelleted, and incubated for 10 minutes in 1ml EBC lysis buffer [Petti, *et al.*, 1991], which is a Tris-buffered 120mM sodium chloride solution containing 0.5% NP40. Debris was pelleted by centrifugation and the supernatants were transferred to a fresh tube and diluted 1:3 in EBC to facilitate sample handling. The protein content in each sample was assayed using Coomassie assay reagent (Pierce), and a volume of lysate containing 2 mg protein was used for each immunoprecipitation.

EGFR was immunoprecipitated with 900 ng of anti-EGFR mAb 528 [Gill, *et al.*, 1984] and 7.2 ug rabbit anti-mouse antibody 31188 (Pierce); neu was immuno-precipitated with 2 ug of anti-Neu mAb TAb 250 [Langton, *et al.*, 1991], and 12 ug 31188 or with 1 ug of anti-Neu mAb FSP-16 [Harwerth, *et al.*, 1992] and 5 ug 31188;

erbB-3 was immunoprecipitated with 200 ng of anti-ErbB-3 rabbit polyclonal antiserum SC-285 (Santa Cruz Biotechnology); erbB-4 was immunoprecipitated with 1ug of anti-ErbB-4 rabbit polyclonal antiserum SC-283 (Santa Cruz Biotechnology). Specificity of anti-receptor antibodies was verified by testing each precipitating antibody for cross-reactivity with cell lines expressing heterologous receptors. All immunoprecipitations were incubated at 4°C for 2 hours, after which the immune complexes were collected by incubation at 4°C with 50ul of a 10% (v/v) suspension of fixed and washed *S. Aureus* (IGSL-10 - The Enzyme Center). Immune complexes were washed 3 times with NET-N [Petti, *et al.*, 1991] and were eluted from *S. Aureus* by boiling in 150ul protein sample buffer [Petti, *et al.*, 1991]. Samples were divided equally, electrophoresed on separate 7.5% acrylamide, 0.17% bisacrylamide, 0.1% SDS gels [Sefton, *et al.*, 1979], and transferred to nitrocellulose [DiGiovanna and Stern, 1995] for immunoblotting with either the antiphosphotyrosine mAb 4G10 (Upstate Biotechnology, Inc.) or antibodies specific for receptors. Antibody binding was detected with sheep anti-mouse coupled to horseradish peroxidase (HRP) antibody NA931 (Amersham) or donkey anti-rabbit coupled to HRP antibody NA934 (Amersham) and enhanced chemiluminescence (ECL) reagents RPN2106 (Amersham). Anti-receptor immunoblotting antibodies were sheep anti-EGFR polyclonal antibody 06-129 (Upstate Biotechnology Inc.) and rabbit anti-sheep antibody 31240 (Pierce); rabbit anti-neu antibody Ab1 (PC04; Oncogene Science); mouse anti-erbB-3 monoclonal antibody 2F12 [Kim, *et al.*, 1994]; and rabbit anti-erbB-4 polyclonal antibody SC-283 (Santa Cruz Biotechnology).

Results

Generation of recombinant Ba/F3 cell lines.

The mouse Ba/F3 pro-B-lymphocyte cell line was chosen for expression of erbB family members because their survival and proliferation is tightly regulated by exogenous growth factor (interleukin-3), and because erbB family receptors have not been found to be expressed in mammalian hematopoietic cells. Immunoprecipitation and immunoblotting experiments did not reveal endogenous expression of any erbB family receptors in these cells (data not shown). Nonetheless, we further assessed endogenous receptor expression by polymerase chain reaction amplification of reverse-transcribed transcripts (RT-PCR assay), the most sensitive assay available. RT-PCR analysis of erbB family receptor transcription using probes homologous to murine erbB family receptor genes in a control Ba/F3 cell line or in Ba/F3 cell lines expressing exogenous human EGFR, neu, erbB-3 or erbB-4 demonstrated that these lines lacked endogenous murine EGFR, neu or erbB-4 transcription (data not shown). Given that the human CEM T-lymphocyte cell line lacked endogenous EGFR, neu, erbB-3, or erbB-4 transcription (data not shown), we were surprised to discover that all of the Ba/F3 cell lines tested exhibited detectable levels of endogenous erbB-3 transcription (data not shown). This novel finding implies that erbB family receptors and their ligands may play important roles in the differentiation, expansion, or growth transformation of cells of a B-lymphocyte lineage.

cDNAs directing the expression of erbB family receptors were introduced into Ba/F3 cells to generate clonal lines that express the four different human receptors, singly and in combination. We first stimulated the resulting panel of cell lines with a chemically-synthesized NRG-β 65-mer peptide (amino acids 177-241 of NRG β1), which encompasses the EGF homology domain and is sufficient for induction of

receptor tyrosine phosphorylation [Holmes, et al., 1992; Barbacci, et al., 1995]. Regulation of tyrosine phosphorylation of each receptor by NRG-β was evaluated by immunoprecipitating the receptors and immunoblotting with anti-phosphotyrosine (Figures 1A and 3A) and anti-receptor antibodies (Figures 1B and 3B).

NRG-β stimulation of erbB receptor tyrosine phosphorylation.

Among cell lines expressing a single exogenous receptor (Figure 1a), NRG-β failed to stimulate tyrosine phosphorylation of the EGFR or erbB-3. In contrast, NRG-β strongly activated tyrosine phosphorylation of erbB-4. Since high basal tyrosine phosphorylation of neu in the neu/5 cell line may have obscured the effect of NRG-β, we isolated an independent Ba/F3 derivative, denoted neu/12C, that expresses considerably less neu than the neu/5 cell line. In this cell line, NRG-β clearly activated neu tyrosine phosphorylation (Figure 2), probably as the result of neu heterodimerization with the endogenous erbB-3 in Ba/F3 cells.

In most of the double recombinant cell lines, NRG-β unambiguously stimulated tyrosine phosphorylation of both erbB family receptors (Figure 3A; summarized in Table 1). Since the four erbB family receptors have distinct electrophoretic mobilities in most combinations, co-precipitation of heterologous dimerization partners would have been detected. However, co-precipitation was not observed under these conditions. Significantly, the results in the double recombinant cell lines are not simply additive with the responses of single cell lines. For example, NRG-β does not stimulate tyrosine phosphorylation of the uniquely expressed EGFR, but exogenous coexpression of erbB-3 or erbB-4 with EGFR enabled NRG-β to regulate EGFR tyrosine phosphorylation. Similarly, while NRG-β did not stimulate tyrosine phosphorylation of erbB-3 alone, coexpression of EGFR, neu, or erbB-4 permitted activation of erbB-3. Thus NRG-β can regulate the tyrosine

phosphorylation of each erbB family receptor provided the appropriate co-receptor is expressed.

Betacellulin, EGF, and NRG-β stimulation of erbB family receptor tyrosine phosphorylation.

We also compared erbB family receptor tyrosine phosphorylation following stimulation of the panel of recombinant Ba/F3 cell lines with recombinant human betacellulin, NRG-β, or EGF. The recombinant human betacellulin used in these experiments consisted of 34 amino acids of the human amphiregulin precursor (Val107 - Arg140), linked to the 50 amino acid EGF-structural motif of human betacellulin (Arg31 - Tyr80) and a 9 amino acid hemagglutinin epitope. The amphiregulin sequences in this molecule are not within the EGF-structural motif and therefore are not predicted to contribute to receptor binding. Furthermore, this recombinant betacellulin produced as a tagged fusion construct in bacteria had an activity indistinguishable from that produced transiently in COS cells using an expression construct containing the full length human betacellulin sequence (Plowman, *et al.*, in preparation).

In cell lines that ectopically express a single erbB family receptor, both betacellulin and EGF stimulated EGFR tyrosine phosphorylation (Figures 4 and 5a). Surprisingly, betacellulin (as well as NRG-β) stimulated erbB-4 tyrosine phosphorylation (Figures 4 and 5a). Not only do these data demonstrate that betacellulin regulates EGFR signaling, but they also indicate that erbB-4 is a receptor for betacellulin. Betacellulin did not stimulate increased neu or erbB-3 tyrosine phosphorylation (Figures 4 and 5a), suggesting that neither neu nor erbB-3 is a receptor for betacellulin (again, NRG-β stimulation of neu tyrosine phosphorylation is probably due to endogenous erbB-3 expression). However, because erbB-3 has

only minimal intrinsic tyrosine kinase activity [Guy, *et al.*, 1994], these data do not rule out the possibility that erbB-3 can bind betacellulin.

In activating both the EGFR and erbB-4, betacellulin displays activities distinct from EGF, which activates the EGFR alone, and NRG-β, which binds to erbB-3 and erbB-4. We next compared the effects of betacellulin, EGF, and NRG-β on receptor transmodulation by assessing receptor tyrosine phosphorylation in cell lines expressing combinations of erbB family receptors. Betacellulin, as well as EGF, stimulated the tyrosine phosphorylation of both receptors in the EGFR + neu (1+2), EGFR + erbB-3 (1+3), and EGFR + erbB-4 (1+4) cell lines (Figures 5b and 5c). Therefore, both betacellulin and EGF can transmodulate the other three receptors when co-expressed with EGFR. However, the three ligands did not stimulate equal levels of receptor phosphorylation. In 1+3 cells, all three ligands stimulated approximately equal levels of erbB-3 phosphorylation, while betacellulin and EGF stimulated higher levels of EGFR phosphorylation than NRG-β did (Figure 5b). Similarly, in 1+4 cells, betacellulin stimulated approximately equal levels of EGFR and erbB-4 phosphorylation, while EGF stimulated higher levels of EGFR phosphorylation than erbB-4 phosphorylation and NRG-β stimulated higher levels of erbB-4 phosphorylation than EGFR phosphorylation (Figure 5c). One explanation for these differences is that EGFR, erbB-3, and erbB-4 may have a lower affinity for heterotypic interactions and transmodulation than for the homotypic interactions induced by direct stimulation.

As expected from the response of the erbB-4 cell line, betacellulin and NRG-β stimulated erbB-4 tyrosine phosphorylation in the three double recombinant cell lines that express erbB-4. However, while betacellulin and NRG-β stimulated EGFR and neu tyrosine phosphorylation in the EGFR + erbB-4 (1+4) and neu + erbB-4 (2+4) cell lines, respectively, betacellulin did not stimulate erbB-3 tyrosine

phosphorylation in the erbB-3 + erbB-4 (3+4) cell line, even through NRG-β did (Figures 5c and 5d). Finally, betacellulin did not stimulate phosphorylation of either receptor in neu + erbB-3 cells, suggesting that neither neu nor erbB-3 is a receptor for betacellulin.

Ligand stimulation of Ba/F3 IL-3 independent survival or proliferation.

While NRG-β, betacellulin, and EGF can all stimulate the tyrosine phosphorylation of all four erbB family receptors, either directly or through transmodulation, activation of different receptors or combinations of receptors may specify unique biological responses through coupling of each receptor to distinct cellular signaling pathways. We investigated this possibility by determining whether ligand stimulation enabled survival or growth of the various Ba/F3 derivatives independent of interleukin-3 (IL-3). Ectopic expression of a number of receptors in Ba/F3 cells permits receptor regulation by the cognate ligands, which in some cases relieves dependence on IL-3 for survival or growth: expression of the erythropoietin receptor with Friend Spleen Focus-Forming Virus gp55 permits IL-3-independent proliferation [Li, *et al.*, 1990]. Similarly, EGF stimulation of Ba/F3 cells expressing exogenous EGFR results in EGFR tyrosine phosphorylation and increased cellular DNA synthesis [Collins, *et al.*, 1988; Shibuya, *et al.*, 1990], while stimulation of Ba/F3 derivatives expressing exogenous platelet derived growth factor (PDGF) receptor with PDGF results in receptor tyrosine phosphorylation and IL-3-independent proliferation [Satoh, *et al.*, 1993].

In the absence of ligand, all of the Ba/F3 derivatives remained dependent on IL-3 for survival, even those lines that display substantial basal receptor tyrosine phosphorylation (Figures 6a and 6b). Activation of either EGFR or neu in the single recombinant cell lines was associated with IL-3 independent survival but not

proliferation (Figure 6a), while activation of erbB-3 or erbB-4 in the single recombinants had no biological effect (Figure 6a). Therefore, ligand stimulation of erbB phosphorylation was necessary, but not sufficient, for an IL-3 independent response (Table 1).

We also assessed ligand activity in the double recombinant Ba/F3 cell lines (Figure 6b). As expected from the responses of the single recombinant cell lines, receptor activation in cells expressing EGFR or neu conferred, with one notable exception, a minimal response of IL-3 independent survival. For example, in EGFR + neu (1+2), EGFR + erbB-4 (1+4), neu + erbB-3 (2+3), and neu + erbB-4 (2+4) cell lines, receptor activation stimulated a minimum of IL-3-independent survival, while in erbB-3 + erbB-4 (3+4) cells none of the ligands stimulated an IL-3 independent response. The exception is the response of 1+3 cells to ligand stimulation. As predicted, betacellulin and EGF stimulated the IL-3 independent survival of 1+3 cells; however, NRG-β failed to stimulate an IL-3 independent response.

In some of the double recombinant cell lines ligand stimulation of coupling of multiple receptors to signaling pathways acted in a non-additive manner to stimulate an IL-3 independent response (Figure 6b). In 1+2 cells betacellulin and EGF stimulated IL-3 independent proliferation, while in 1+4 cells betacellulin and NRG-β stimulated IL-3 independent proliferation and EGF stimulated a response intermediate to survival and proliferation. Therefore, while activation of either EGFR alone or neu alone stimulated IL-3 independent survival, in some cases activation of EGFR along with either neu or erbB-4 conferred IL-3 independent proliferation.

Conclusions/Discussion

Discussion

We have analyzed the NRG-dependent responses conferred upon the Ba/F3 mouse pro-B-lymphocyte cell line by expression of the four erbB family receptors, singly and in pairwise combinations. In the presence of appropriate co-receptors, NRG regulated the tyrosine phosphorylation of all four erbB family receptors. While some of the NRG-induced interactions between erbB family receptors observed had been predicted from previous work, we demonstrate here for the first time that in the presence of erbB-3 or erbB-4, NRG regulates tyrosine phosphorylation of the EGFR, and that the presence of the EGFR, neu, or erbB-4 enables NRG to regulate tyrosine phosphorylation of erbB3. Furthermore, NRG induces IL-3-independent survival or proliferation in only a subset of the lines that exhibit NRG-induced receptor tyrosine phosphorylation. Thus the biological responses to NRG are specified at several different levels of regulation.

Previous analyses of NRG-induced signaling by erbB family receptors have been carried out with a few receptor combinations in a variety of cell backgrounds. In mammary cells, NRGs induce neu tyrosine phosphorylation, can be cross-linked to neu, and binding is increased by neu overexpression [Peles, *et al.*, 1993], at first suggesting that neu is a receptor for NRG. However, NRG fails to induce neu tyrosine phosphorylation and/or bind neu when neu is expressed in fibroblasts, ovarian cells [Peles, *et al.*, 1993], CHO cells [Culouscou, *et al.*, 1993; Plowman, *et al*, 1993b], T-lymphoid cells [Plowman, *et al.*, 1993b], or COS-7 cells [Sliwokowski, *et al.*, 1994], and NRG does not bind to solubilized neu extracellular domains [Tzahar, *et al.*, 1994]. Moreover, NRG binds erbB-3 [Carraway, *et al.*, 1994; Kita, *et al.*, 1994; Sliwkowski, *et al.*, 1994; Tzahar, *et al.*, 1994] or erbB-4 [Culouscou, *et al.*, 1993; Plowman, *et al.*, 1993a; Plowman *et al.*, 1993b; Tzahar, *et al.*, 1994], and co-expression

of erbB-3 or erbB-4 with neu confers NRG responsiveness upon neu, probably through the formation of neu/erbB-3 or neu/erbB-4 heterodimers [Carraway, *et al.*, 1994; Kita, *et al.*, 1994; Plowman *et al.*, 1993b; Sliwkowski, *et al.*, 1994]. This has led to the general working hypothesis that activation of neu by NRG requires the presence of erbB-3 or erbB-4.

The present data are compatible with this conclusion, and extend the model to include NRG regulation of the EGFR. The EGFR and erbB-3 alone fail to respond to NRG for two different reasons. The EGFR does not bind NRG [Holmes, *et al.*, 1992], whereas erbB-3 binds, but is impaired for kinase activity [Guy, *et al.*, 1994]. The stimulation of tyrosine phosphorylation of neu by NRG might suggest direct activation of neu by NRG, but in view of previously published work, is more likely to reflect interaction with endogenous erbB-3. However, erbB-4 is able to bind and respond to NRG directly.

NRG induces extensive cross-talk among receptors expressed in binary combinations (Table 1). Either erbB-3 or erbB-4, both of which bind NRG, enable regulation of the EGFR by NRG. This is the first evidence that NRG can regulate EGFR signaling. As predicted from earlier work, NRG stimulates tyrosine phosphorylation of both receptors in the neu + erbB3, neu + erbB-4, and erbB-3 + erbB-4 cell lines. Coexpression of EGFR, neu, or erbB-4 with erbB-3 permits NRG induction of erbB-3 tyrosine phosphorylation. Although earlier work showed that expression of neu enhances tyrosine phosphorylation and NRG regulation of erbB-3 [Carraway, *et al.*, 1994], that work was done in COS-7 cells, which express significant basal amounts of neu and EGFR. Thus the present work demonstrates for the first time that *de novo* expression of either the EGFR, neu, or of erbB-4 enables hormone-regulated phosphorylation of erbB-3. Endogenous erbB receptor expression in Ba/F3 cells played a limited, yet significant role in specifying responses

to NRG stimulation in these experiments. While endogenous erbB-3 expression permits NRG stimulation of exogenous neu tyrosine phosphorylation, NRG does not stimulate receptor tyrosine phosphorylation in cells that express exogenous EGFR only. Perhaps the level of endogenous erbB-3 expression in Ba/F3 cells is insufficient to permit NRG-induced EGFR tyrosine phosphorylation. Alternatively, intrinsic differences between the exogenous human and endogenous mouse proteins may result in the differing capacities to undergo NRG-induced heterotypic receptor interactions.

While NRG can stimulate the tyrosine phosphorylation of each receptor under the appropriate conditions, the diversity of biological responses to NRG indicates that there must be additional mechanisms by which biological responses to NRG are specified. The patterns of NRG-induced stimulation of erbB receptor tyrosine phosphorylation and IL-3 independent survival or proliferation demonstrates that there are several hierarchical levels at which biological responses to NRG are apparently specified.

First, responsiveness to NRG requires the expression of erbB-3 or erbB-4. Previous work and results presented here establish that NRG can not bind or stimulate tyrosine phosphorylation of erbB family receptors in the absence of erbB-3 or erbB-4 expression. Biological responses to NRG are also specified by the intrinsic kinase activity of the erbB family receptor(s) stimulated by NRG, since the kinase-deficient erbB-3 requires the presence of a co-receptor for hormone-regulated phosphorylation.

Distinct biological responses to NRG are also conferred by ligand-induced coupling of different erbB family receptors to different signaling pathways. NRG enables the IL-3 independent survival of cell lines expressing neu, probably through NRG-induced activation of neu via erbB-3. However, NRG does not enable the IL-3

independent survival of EGFR + erbB-3 cells, or of erbB-4 cells, even though NRG stimulates receptor tyrosine phosphorylation in these lines. This demonstrates that neu has signaling properties distinct from those of the EGFR, erbB-3, or erbB-4, and is consistent with earlier work showing that different erbB family receptors can activate different signaling pathways and responses [DiFiore, et al, 1990; Fedi, et al., 1994; Kim, et al., 1994; Prigent and Gullick, et al., 1994; Soltoff, et al., 1994; Carraway, et al., 1995]. Neither NRG nor EGF induces IL-3-independent proliferation of cells that individually express erbB-4 or EGFR (Table 1 and Figure 6a). Yet, NRG stimulates IL-3-independent proliferation in the EGFR + erbB-4 cell line. One simple explanation would be that IL-3-independent proliferation requires activation of two independent pathways, one of which is activated by the EGFR, and one by erbB-4. An interesting alternative would be that EGFR and erbB-4 phosphorylation sites differ in ligand-induced EGFR/erbB-4 heterodimers than in ligand-induced receptor homodimers owing to substrate specificity of the receptor catalytic domains and steric considerations in the cross-phosphorylation reaction. This would permit recruitment of unique signaling proteins to the heterodimer, resulting in unique biological responses. Thus, the diversity of hormone-regulated outputs from this receptor outwork may extend beyond the simple combinatorial possibilities.

Since EGF and NRG bind to different receptors, it can be predicted that individual members of the EGF family of ligands activate different constellations of erbB family receptors, so that these different ligands will yield distinct patterns of biological responses. Evidence presented here supports this prediction. Previous reports demonstrated that betacellulin binds to the A431 human adenocarcinoma cell line and the MDA-MB-453 human breast carcinoma cell line, both of which overexpress the EGFR. This binding was quenched by the addition of an excess of EGF, suggesting that betacellulin is a ligand for the EGFR [Watanabe, *et al.*, 1994].

However, the effect of betacellulin on EGFR tyrosine phosphorylation and signaling and the possibility that betacellulin might activate other erbB family receptors were not assessed. Here we show that in Ba/F3 cells expressing only a single ectopic erbB family receptor, betacellulin stimulates the tyrosine phosphorylation of both the EGFR and, surprisingly, erbB-4 (Table 1). This is consistent with the observation that radiolabeled betacellulin binds specifically to EGFR and erbB-4, but not to neu (Plowman, *et al.*, in preparation). Control experiments performed in parallel demonstrated that radiolabeled amphiregulin and EGF bound only to EGFR and radiolabeled NRG-β bound only to erbB-4, as previously reported. Thus, betacellulin exhibits activities that are distinct from those displayed by EGF, which activates the EGFR alone, and NRG-β, which activates erbB-3 and erbB-4 (Table 1). Furthermore, in this first comprehensive analysis of erbB family transmodulation for both betacellulin and EGF, we find that EGF can transmodulate erbB-4 in the EGFR + erbB-4 cell line (Table 1). We also demonstrate that betacellulin stimulates a pattern of receptor transmodulation that is qualitatively distinct from the patterns stimulated by EGF and NRG-β.

With one exception, betacellulin, EGF, and NRG-β transmodulated the tyrosine phosphorylation of all four erbB family receptors in cell lines that express any receptor for each ligand (Table 1). Thus, differences in ligand activities in the double recombinant cell lines can be predicted by differences in activities in the single recombinant cell lines. For example, NRG-β activates erbB-3, while betacellulin does not activate neu or erbB-3. Not surprisingly, in cells expressing neu + erbB-3, NRG-β stimulates the phosphorylation of both receptors, while betacellulin does not stimulate the phosphorylation of either receptor in this cell line (Table 1). The single exception is the response of the erbB-3 + erbB-4 (3+4) cell line to betacellulin. Both betacellulin and NRG-β stimulate erbB-4 tyrosine

phosphorylation in the single recombinant cell line. However, in 3+4 cells, NRG-β stimulates the tyrosine phosphorylation of both receptors, while betacellulin stimulates the tyrosine phosphorylation of erbB-4 but not of erbB-3 (Table 1). Nonetheless, because NRG-β binds erbB-3, it is not clear that this absence of erbB-3 tyrosine phosphorylation is due to differences between betacellulin- or NRG-β-induced erbB-3 transmodulation.

Previous work demonstrated that different erbB family receptors or combinations of receptors couple to distinct cellular signaling pathways. For example, EGFR activation stimulates the tyrosine phosphorylation of four proteins that are not highly phosphorylated following neu activation [Fazioli, *et al.*, 1992]. Furthermore, activated erbB-3 stimulated higher levels of phosphatidylinositol 3-kinase than EGFR did [Fedi, *et al.*, 1994; Soltoff, *et al.*, 1994; Carraway, *et al.*, 1995], and it has been suggested that EGFR and neu bind the adapter protein GRB2, but erbB-3 does not [Prigent and Gullick, 1994; but also see Kim, *et al.*, 1994; Fedi, *et al.*, 1994]. These different coupling capacities of the erbB family receptors can be correlated to specific biological responses. Activation of the EGFR stimulates the IL-3 independent proliferation of 32D myeloid cells, while wild-type and mutationally-activated neu alleles do not [DiFiore, *et al.*, 1990]. In Ba/F3 cells, however, activation of neu stimulates IL-3 independent survival, while activation of EGFR and erbB-4 together stimulates IL-3 independent proliferation [Riese, *et al.*, submitted].

We found that betacellulin stimulates IL-3 independent survival or proliferation in neu + erbB-4 cells and in every cell line that expresses EGFR. In contrast, EGF stimulated IL-3 independence only in those cell lines that express EGFR while NRG-β stimulated IL-3 independence only in the EGFR + erbB-4 cell line and in those cell lines that express neu (Table 1). Therefore, with a single exception, the minimal requirement for IL-3 independence is activation of either

EGFR or neu. The exception is that betacellulin and EGF, but not NRG-β, stimulated IL-3 independent survival in the EGFR + erbB-3 cell line (Table 1). This lack of response to NRG-β may merely reflect the lower level of EGFR phosphorylation stimulated by NRG-β in this cell line (Figure 2b). On the other hand, the absence of biological response may reflect the different mechanism by which the EGFR is activated. We demonstrated previously that coupling of these multiple receptor species to cellular signaling pathways acts in a non-additive manner in specifying biological responses [Riese, *et al.*, submitted]. Accordingly, while activation of EGFR or neu by themselves stimulated IL-3 independent survival, activation of EGFR and either neu or erbB-4 together stimulated IL-3 independent proliferation (Table 1).

As we discussed earlier, biological responses to EGF family ligands are regulated by several hierarchical mechanisms. Some, but not all, of these mechanisms are shared by other networks of receptor tyrosine kinases and their ligands, including the neurotrophin network and the fibroblast growth factor (FGF) network. Like the EGF family, the neurotrophin and FGF ligand families have several members that can each activate multiple receptors. The neurotrophin ligand family includes nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4, also known as NT-4/5 or NT-5 [Reviewed in Barbacid, 1994], while the FGF family has at least 9 members encoded by different genes [Reviewed in Johnson and Williams, 1994]. Furthermore, like the erbB receptor family, both the FGF receptor and neurotrophin receptor families have multiple members (FGFR-1, FGFR-1, FGFR-3, FGFR-4 and TrkA, TrkB, TrkC, respectively). Moreover, like the EGFR and erbB-4, some of these FGFRs and Trks can bind multiple ligands [Reviewed in Johnson and Williams, 1994; Barbacid, 1994].

Another regulatory mechanism common to the EGF/erbB and FGF signaling networks is that both use heparan sulfate proteoglycans (HSPGs) to modulate receptor-ligand interactions. FGFs bind with low affinity in a multivalent manner to HSPGs, causing ligand oligomerization [Reviewed in Lemmon and Schlessinger, 1994] and increasing their binding affinity for FGFRs [Reviewed in Eckenstein, 1994]. Because the FGF/FGFR complex exists in a 1:1 stoichiometry [Spivak-Kroizman, *et al.*, 1994], yet FGFs are monomeric, it has been proposed that HSPG binding potentiates FGF stimulation of FGFR phosphorylation and dimerization. HSPGs also regulate the interactions of EGF family ligands with their receptors. Several EGF family ligands bind HSPGs, including NRGs, amphiregulin (AR), and heparin-binding-EGF-like growth factor (HB-EGF), and this binding regulates ligand-receptor interactions [Aviezer and Yayon, 1994; Johnson and Wong, 1994; Cook, *et al.*, 1995a; Cook, *et al.*, 1995b]. However, many of the mechanistic details of regulation by HSPGs have yet to be elucidated.

While the neurotrophin and FGF networks have regulatory mechanisms that are also features of the EGF/erbB network, there are also features of the neurotrophin and FGF networks that are not part of the EGF/erbB network. Alternative splicing produces truncated FGFR and Trk isoforms lacking the cytoplasmic tyrosine kinase domain and sites for tyrosine phosphorylation [Reviewed in Johnson and Williams, 1994; Barbacid, 1994. Therefore, a regulatory mechanism not observed in the EGF/erbB network results in dominant negative receptors, which are not a characteristic of the EGF/erbB network. Another feature that is characteristic of the neurotrophin network and not seen in the EGF/erbB network is regulation by a low-affinity co-receptor. p75, the low-affinity neurotrophin receptor, has no tyrosine kinase domain [Reviewed in Chao, 1994] and p75 binding is in some cases dispensable for biological response [Reviewed in Ibanez,

1994]. Nonetheless, it has been proposed that p75 regulates the biological response to neurotrophins by altering the affinity of neurotrophin binding to the Trk family receptors [Benedetti, *et al.*, 1993; Reviewed in Chao, 1994].

Data presented here suggests that differences in NRG-β, EGF, and betacellulin activities play a significant role in specifying the proliferation and differentiation of human tissues *in vivo*. These ligands may play their most significant role in the mammary epithelium and tissues of neuroectodermal origin. Not only has the expression of EGF family ligands been documented in these cell types, but these ligands can regulate the proliferation and/or differentiation of these cell types in cultured cell or animal model systems. Furthermore, mounting evidence suggests that increases in the expression and/or signaling of erbB family receptors plays a significant role in tumors of mammary or neuroectodermal origin [Reviewed in Hynes and Stern, 1994]. Because betacellulin, NRG-β, and EGF have distinct biological activities that apparently reflect their differing abilities to activate receptor signaling, it may be possible to develop antagonists that specifically disrupt signaling by a single EGF family ligand and may inhibit the genesis or growth of malignancies without disrupting the activity of other EGF family ligands in the same tissue.

Assessment of Proposal Technical Objectives and Ongoing and Future Experiments

A. Subclone erbB receptor and neuregulin cDNAs into retrovirus-based expression vectors and generate recombinant retroviral stocks to facilitate gene transfer and expression.

As described in the "Materials and Methods" section of this report, we have subcloned the four different erbB family receptor cDNAs into the pLXSN recombinant retrovirus-based expression vector. While we have packaged these constructs into recombinant retroviral stocks, the stocks were not used to generate the Ba/F3 derivatives described elsewhere in this report.

B. Develop cultured cell systems for expression and purification of recombinant neuregulin.

We have introduced a neuregulin-α expression vector into mouse C127 fibroblasts, generating cell lines that stably express and secrete neuregulin-α. We have also engineered recombinant baculovirus stocks containing a neuregulin-α cDNA. However, we have not conclusively determined that infection of insect cells with these stocks results in the production of biologically active neuregulin-α. Dr. Frank Jones, a postdoctoral fellow in the Stern Laboratory, is continuing these efforts to produce biologically active recombinant neuregulin.

To bypass these difficulties in producing recombinant neuregulin, we established a collaboration with Drs. James D. Moyer, Brad C. Guarino, and Glenn C. Andrews, Pfizer Central Research, Groton, CT. As described in the "Materials and Methods" section of this report, they have supplied us with NRG-β as a refolded, biologically active, chemically-synthesized 65-mer peptide corresponding to amino acids 177 to 241 of the NRG β1 isoform.

We also established a collaboration with Drs. Sharon Buckley and Gregory D. Plowman, Sugen, Inc., Redwood City, CA. As described in the "Materials and Methods" section of this report, they have supplied us with human recombinant betacellulin as a refolded, biologically active peptide expressed in *E. coli*. They are also supplying us with human recombinant amphiregulin (AR). We have also established collaborations with Dr. Michael Klagsbrun (Children's Hospital, Boston, MA), who is supplying us with recombinant heparin-binding EGF-like growth factor (HB-EGF) and with Dr. David Salomon (NIH/NCI), who is supplying us with cripto. Experiments to compare the activities of HB-EGF, AR, cripto, and transforming growth factor alpha (TGFα - purchased from commercial sources) are currently underway.

C. Determine what erbB receptors are neuregulin effectors in Ba/F3 cells

As described in this report and summarized in Table 1, neuregulin-β (NRG-β) stimulates erbB-4 tyrosine phosphorylation. Similarly, NRG-β stimulates erbB-3 tyrosine phosphorylation, but only when erbB-3 is coexpressed with another erbB family receptor. Furthermore, NRG-β stimulates EGFR and neu tyrosine phosphorylation when these receptors are coexpressed with either erbB-3 or erbB-4. As summarized in Table 1, EGF stimulates EGFR tyrosine phosphorylation, as well as the phosphorylation of any other erbB family receptor when it is coexpressed with the EGFR. Surprisingly, betacellulin stimulates both EGFR and erbB-4 tyrosine phosphorylation. Furthermore, betacellulin stimulates both neu and erbB-3 tyrosine phosphorylation when these receptors are coexpressed with the EGFR. In contrast, betacellulin stimulates neu but not erbB-3 tyrosine phosphorylation when these receptors are coexpressed with erbB-4.

Therefore, NRG-β, EGF, and betacellulin all stimulate distinct patterns of erbB family receptor tyrosine phosphorylation, which may in part account for their differential biological activities.

We are currently extending these findings by characterizing the signaling pathways that are specifically coupled to ligand-activated erbB family receptors. While NRG, betacellulin, and EGF all stimulate tyrosine phosphorylation of both receptors in the EGFR + erbB-3 (1+3) cell line, the IL-3-independent responses of the 1+3 cells to NRG stimulation is markedly different from the responses to EGF and betacellulin stimulation. This suggests that NRG stimulates the coupling of EGFR and erbB-3 to different signaling pathways than do EGF or betacellulin. Because these ligands and their signaling pathways may play a causative role in breast carcinogenesis, the identification of signaling pathways specifically activated by these ligands is a prioritized focus of this project.

D. Determine if neuregulin acts as an adhesion molecule or receptor for erbB proteins.

These experiments are being performed by Jonathan McMenamin-Balano, a predoctoral student in the Stern Laboratory.

E. Assess effector-specific or presentation specific neuregulin-induced protein tyrosine phosphorylation.

These experiments are being performed by Jonathan McMenamin-Balano, a predoctoral student in the Stern Laboratory.

F. Determine if neuregulin-induced erbB receptor signaling confers IL-3 independent growth to Ba/F3 cells.

As illustrated in Figures 6a and 6b and summarized in Table 1 and as we have discussed in detail elsewhere, neuregulin (NRG) stimulates IL-3-

independent survival of any Ba/F3 derivative that expresses neu. Furthermore, NRG stimulates IL-3-independent proliferation of the EGFR + erbB-4 (1+4) cell line. This pattern of responses to NRG stimulation differs from the patterns of responses to EGF or betacellulin stimulation.

G. *Determine if neuregulin-induced erbB receptor signaling is capable of transforming the growth or altering the differentiative capacity of MCF-10A cells.*

We are currently establishing conditions for assaying ligand activity in MCF-10A cells.

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Appendices

Table 1. *Summary of Stimulation of Receptor Tyrosine Phosphorylation and IL-3 Independence*

Cell Line	Receptor	Betacellulin Tyr Phos.[a]	Betacellulin IL-3 Indpt.[b]	NRG-β Tyr Phos.[a]	NRG-β IL-3 Indpt.[b]	EGF Tyr Phos.[a]	EGF IL-3 Indpt.[b]
EGFR		+	S	-	N	+	S
Neu		-	N	+[c]	S[c]	-	N
erbB-3		-	N	-	N	-	NT[d]
erbB-4		+	N	+	N	-	NT[d]
EGFR+Neu	EGFR	+	P	*[c]	S[c]	+[e]	P
	Neu	+		*[c]		+[e]	
EGFR+erbB-3	EGFR	+	S	+	N	+	S
	erbB-3	+		+		+	
EGFR+erbB-4	EGFR	+	P	+	P	+	S/P
	erbB-4	+		+		+	
Neu+erbB-3	Neu	-	N	+	S	-	NT[d]
	erbB-3	-		+		-	
Neu+erbB-4	Neu	+	S	+	S	-	N
	erbB-4	+		+		-	
erbB-3+erbB-4	erbB-3	-	N	+	N	-	N
	erbB-4	+		+		-	

[a]Results are abstracted from Figures 1-5, and similar unpublished data. "+" indicates increased receptor tyrosine phosphorylation over basal levels, "-" indicates no increase in receptor tyrosine phosphorylation, and "*" indicates ambiguity due to high basal levels of receptor phosphorylation. On this table the "Neu" cell line refers to the neu/12C cell line.

[b]Results are abstracted from Figure 6. "N" indicates no IL-3 independent response, "S" indicates stimulation of IL-3 independent survival, "P" indicates stimulation of IL-3 independent proliferation, and "S/P" indicates stimulation of an intermediate response. "NT" indicates not tested.

[c]The NRG-β response is apparently due to interactions with the endogenous erbB-3 in Ba/F3 cells.

[d]Given the absence of receptor tyrosine phosphorylation, no IL-3 independent response is expected.

[e]Expected from previously published results [Akiyama, *et al.*, 1988; King, *et al.*, 1988; Stern and Kamps, 1988; Connelly and Stern, 1990].

Figures 1a,b. *Regulation of receptor tyrosine phosphorylation by NRG-β in single recombinant Ba/F3 derivatives.*

Untreated or NRG-stimulated cell lines were immunoprecipitated with anti-receptor antibodies and portions of immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine (Panel A) or anti-receptor (Panel B) antibodies. V refers to LXSN (vector only) cells. The "Neu" line used is neu/5. Immunoprecipitating antibodies were: α1, anti-EGFR; α2, anti-Neu antibody; α3, anti-erbB-3; α4, anti-erbB-4; N, normal mouse or rabbit serum. Immunoprecipitations of lysates from NRG-treated cells are denoted by "+" while immunoprecipitations of lysates from untreated cells are denoted by "-".

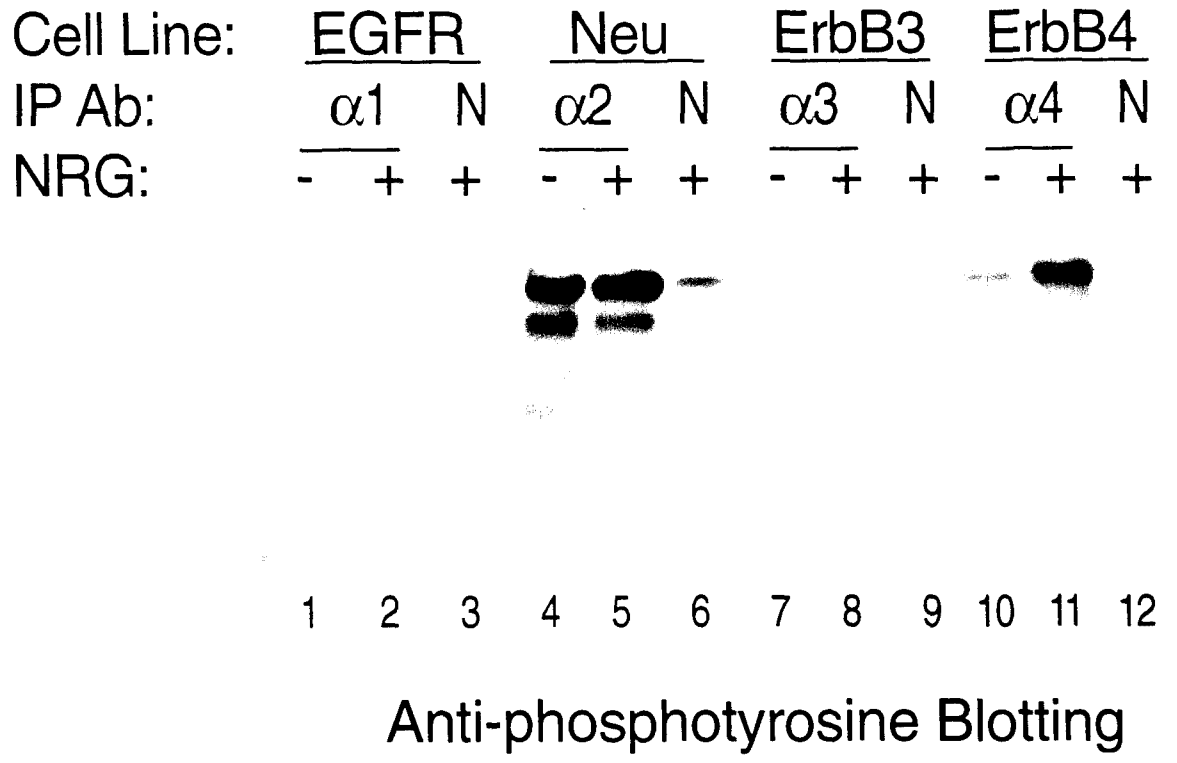

Figure 1a

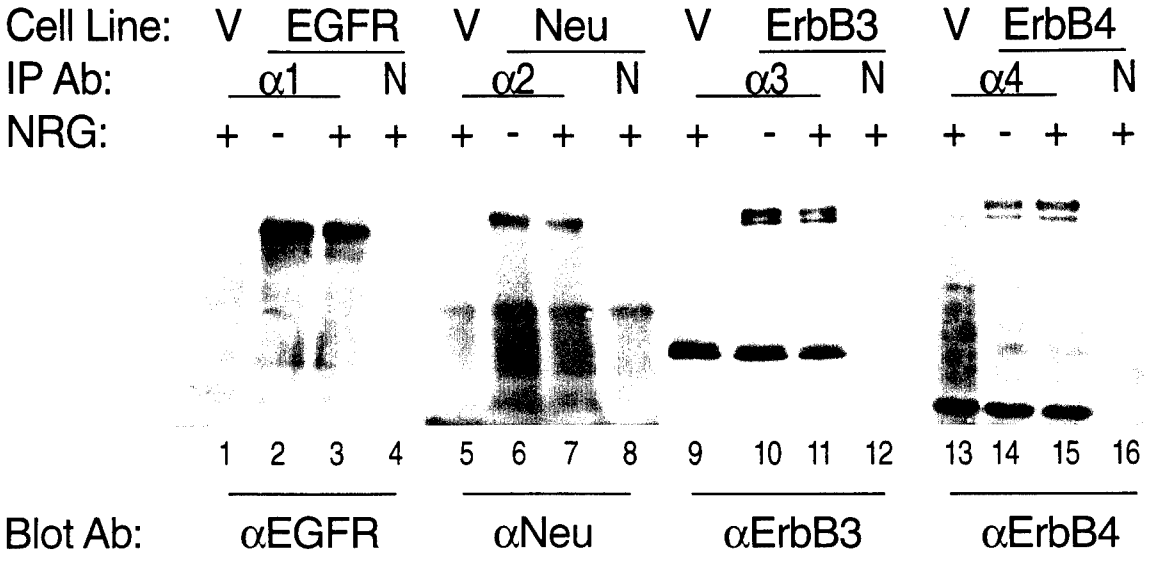
Cell Line: V EGFR V Neu V ErbB3 V ErbB4
IP Ab: α1 N α2 N α3 N α4 N
NRG: + - + + + - + + + - + + + - + +
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Blot Ab: αEGFR αNeu αErbB3 αErbB4

Figure 1b

Figure 2. *Regulation of neu/12C cells by NRG-β.*

neu/12C cells were incubated with NRG dilution buffer ("-"), the agonistic anti-neu antibody TAb 250 [Shawver, *et al.*, 1994] ("A"), or NRG-β ("N"). Lysates were immunoprecipitated with anti-neu antibody ("α2") or normal mouse serum ("N") and analyzed by immunoblotting with anti-phosphotyrosine. Ligand-induced erbB family receptor heterodimers have not been detected by co-immunoprecipitation in the absence of cross-linking agents. Therefore, it is unlikely that the additional band in lane 3 representing a high molecular weight tyrosine phosphorylated protein is endogenous erbB-3. Because this band was not seen in other trials, it is likely that the band represents an antibody-neu complex that is the result of incomplete sample reduction.

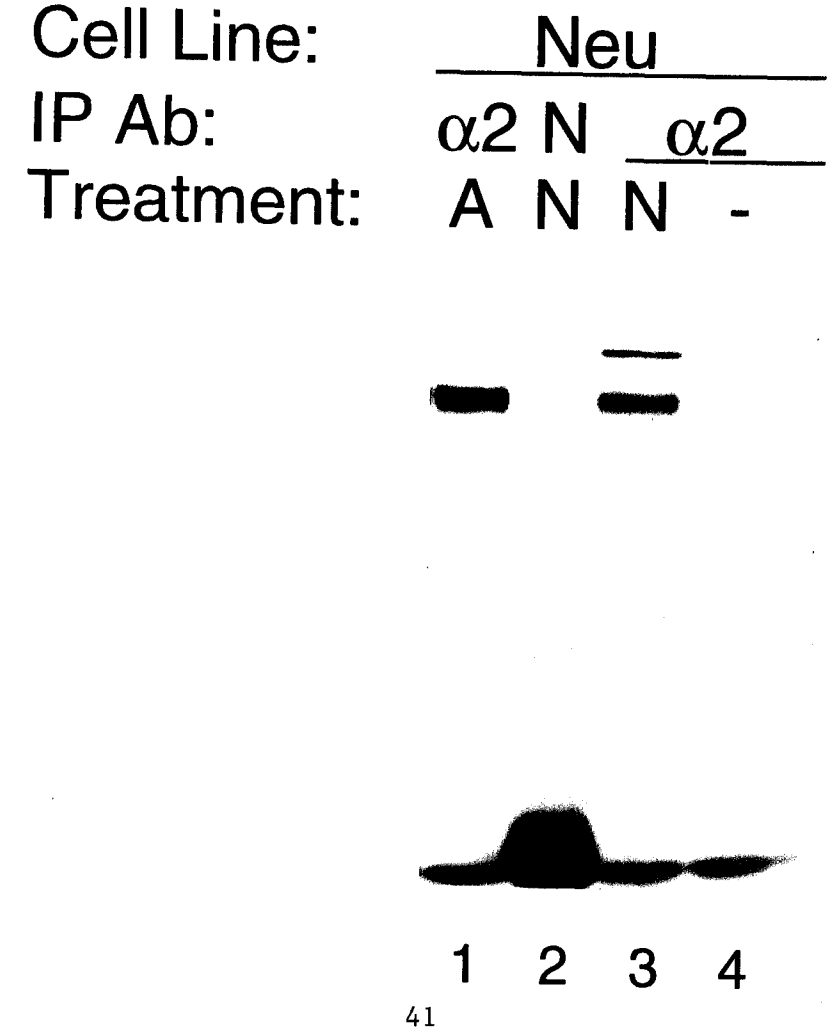

Figure 2

Figures 3a,b. *NRG -β Regulation of receptor tyrosine phosphorylation in double recombinant Ba/F3 derivatives.*

Untreated or NRG-β-treated cells were immunoprecipitated with anti-receptor antibodies and analyzed by immunoblotting with anti-phosphotyrosine (Panel a) or anti-receptor (Panel b) antibodies. Cell lines are abbreviated: 1+2, cell line expressing EGFR + neu; 1+3, EGFR + erbB-3; 1+4, EGFR + erbB-4; 2+3, neu + erbB-3; 2+4, neu + erbB-4; 3+4, erbB-3 + erbB-4. The immunoprecipitating and/or immunoblotting antibodies were: α1, anti-EGFR antibody; α2, anti-neu antibody; α3, anti-erbB-3 antibody; α4, anti-erbB-4 antibody. Immunoprecipitations of lysates from NRG-β-treated cells are denoted "+" while immunoprecipitations of lysates from untreated cells are denoted by "-".

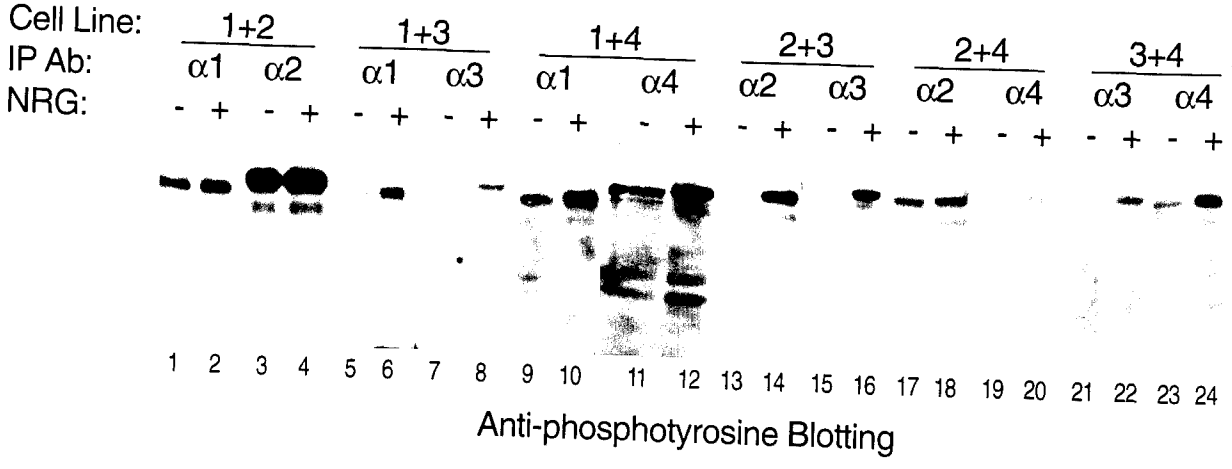

Figure 3a

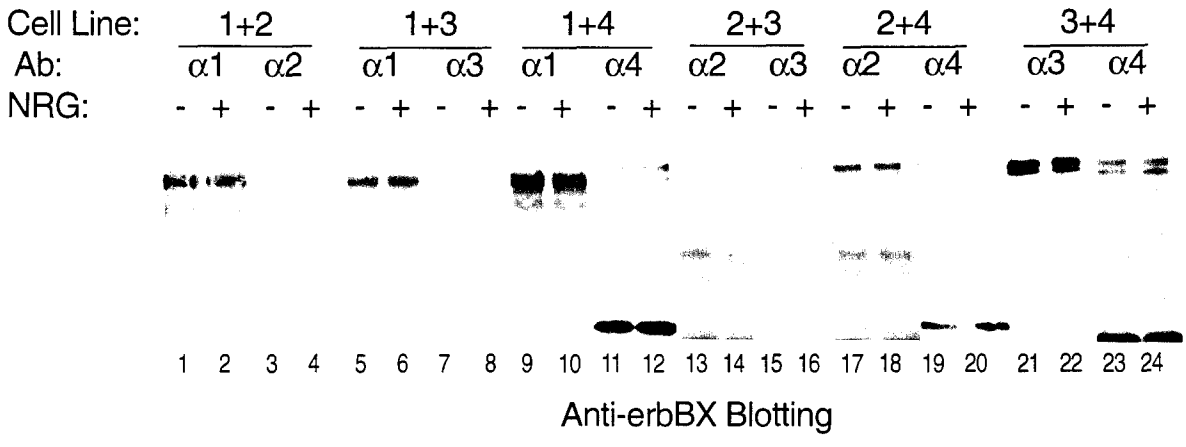
Cell Line: 1+2 1+3 1+4 2+3 2+4 3+4
Ab: α1 α2 α1 α3 α1 α4 α2 α3 α2 α4 α3 α4
NRG: - + - + - + - + - + - + - + - + - + - + - + - +
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Anti-erbBX Blotting

Figure 3b

Figure 4. *Regulation of receptor tyrosine phosphorylation by betacellulin in single recombinant Ba/F3 derivatives.*

Lysates from untreated or betacellulin-treated Ba/F3 derivatives were immunoprecipitated with anti-receptor antibodies and analyzed by immunoblotting with anti-phosphotyrosine antibody. The cell lines and immunoprecipitating antibodies are as marked. Lysates from betacellulin-treated cells are denoted "+" while lysates from untreated cells are denoted "-". The neu cell line used in this experiment is the neu/12C cell line.

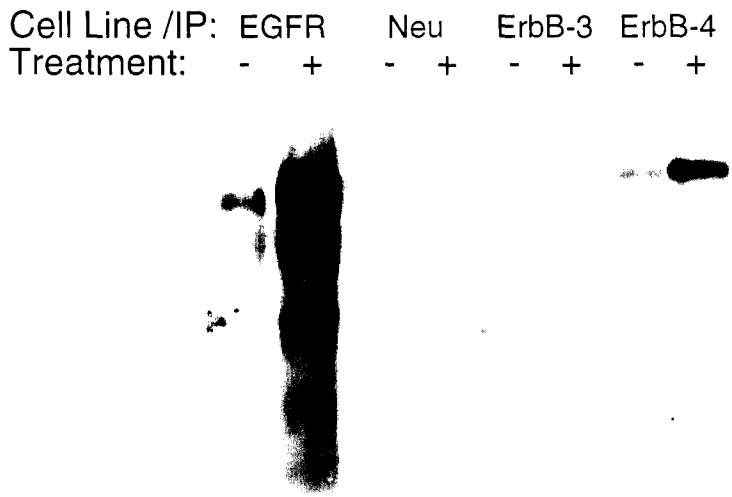

Figure 4

Figures 5a-d. *Comparison of receptor tyrosine phosphorylation in Ba/F3 derivatives stimulated with betacellulin, EGF, or NRG-β.*

Lysates from untreated or ligand-treated Ba/F3 derivatives were immunoprecipitated with anti-receptor antibodies and analyzed by immunoblotting with anti-phosphotyrosine antibody. The cell lines are as marked. Immunoprecipitating antibodies were: α1 or 1, anti-EGFR; α2 or 2, anti-Neu; α3 or 3, anti-erbB-3; α4 or 4, anti-erbB-4. Lysates from betacellulin-treated cells are denoted "B", lysates from NRG-β-treated cells are denoted "N", lysates from EGF-treated cells are denoted "E", and lysates from untreated cells are denoted "-". The neu cell line used in this experiment is the neu/12C cell line.

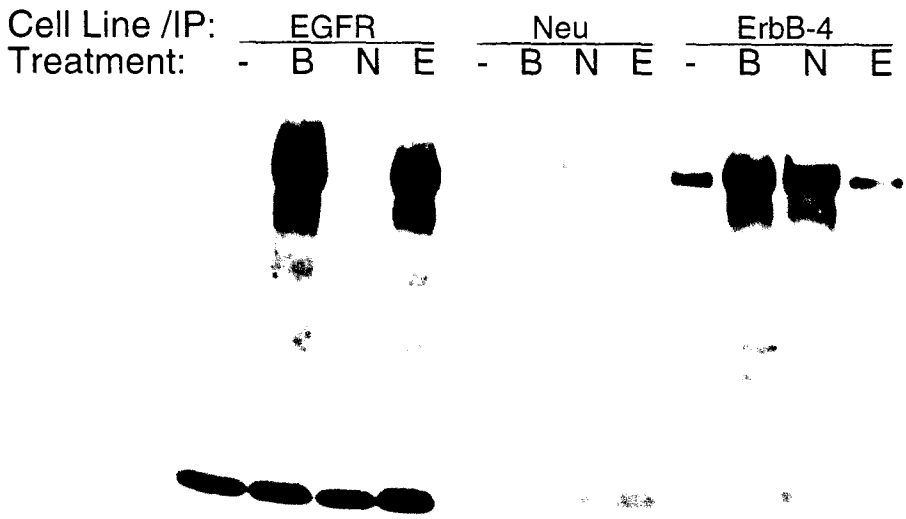

Figure 5a

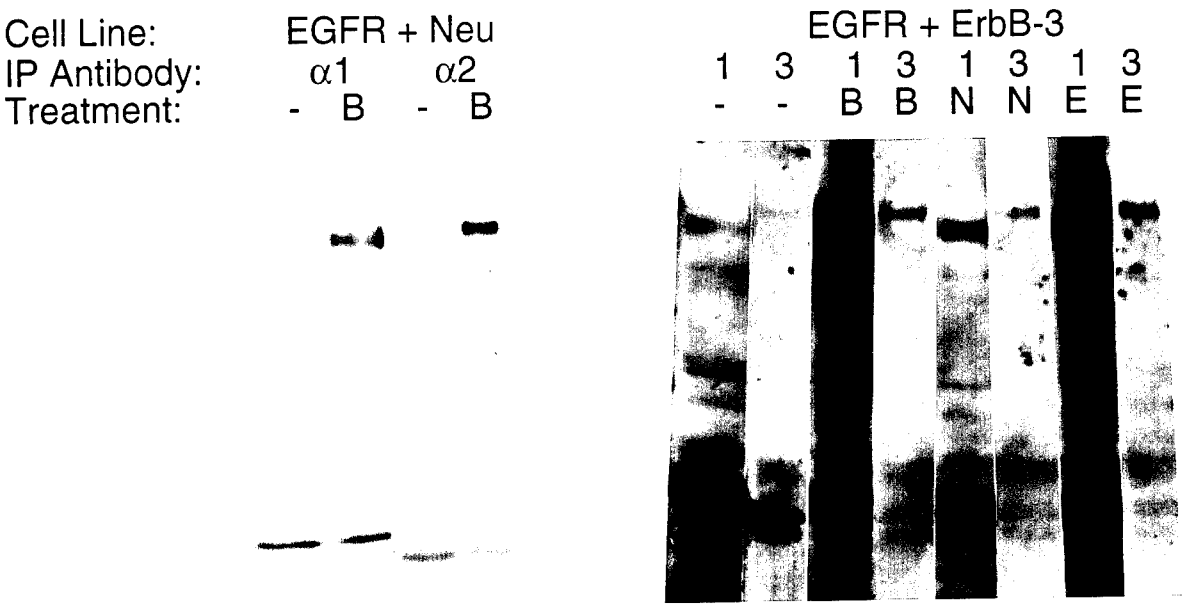

Figure 5b

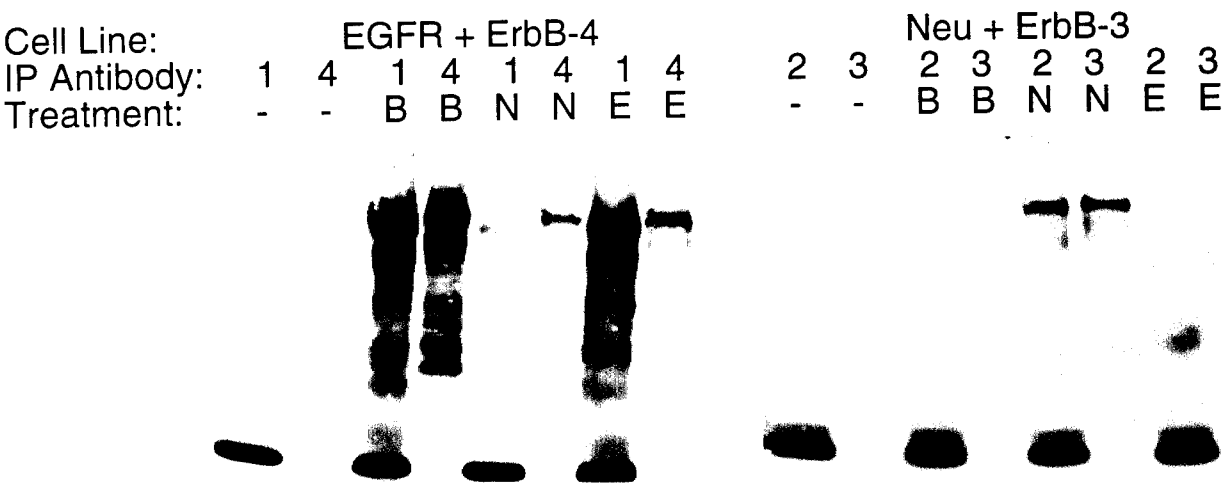

Figure 5c

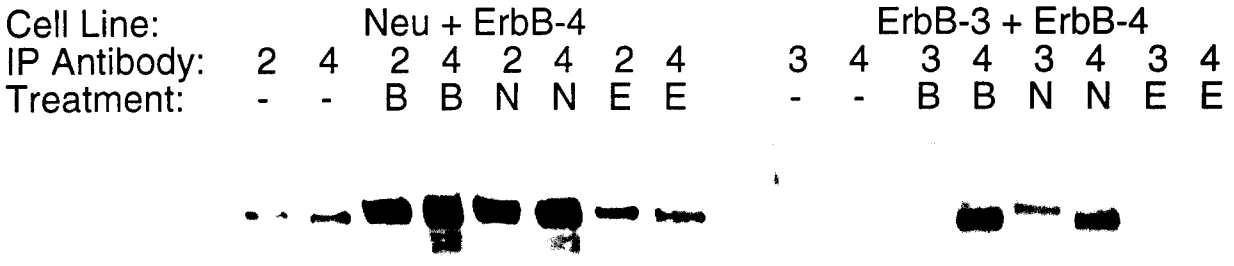
Cell Line:
Neu + ErbB-4
ErbB-3 + ErbB-4
IP Antibody: 2 4 2 4 2 4 2 4 3 4 3 4 3 4 3 4
Treatment: - - B B N N E E - - B B N N E E

Figure 5d

Figures 6a-b. *IL-3-independent saturation density of Ba/F3 cells treated with betacellulin, EGF, or NRG-β.*

For each trial and treatment, Ba/F3 derivatives made quiescent by growth to saturation density were plated at a density of $100x10^3$ cells/ml in culture dishes containing medium lacking IL-3 (IL-3-Free), medium supplemented with IL-3 (IL-3), or in medium lacking IL-3 but supplemented with 10 ng/ml human recombinant EGF (EGF - Collaborative Biomedical), 9.4ng/ml chemically-synthesized NRG-β 65-mer (Neuregulin - [Barbacci, *et al.*, 1995]), or 7ng/ml human recombinant betacellulin (Betacellulin). Cells were stained daily with trypan blue and counted in a hemacytometer to determine viable cell densities until each sample reached a viable cell saturation density, at which time data collection was terminated. While not every cell line was tested with every factor in every trial, each combination of cell lines and factors shown was tested in a minimum of 4 trials, with some combinations being tested in as many as 20 trials. The arithmetic means of the viable cell saturation densities are indicated by the filled bars, while the standard error of the means [Zar, 1984] are indicated by the error bars. Cultures exhibiting viable cell saturation densities of less than $20x10^3$ cells/ml were judged to be nonresponsive to stimulation. Cultures with viable cell saturation densities of 50-$400x10^3$ cells/ml were judged to be exhibiting survival but not proliferation. Cultures with viable cell saturation densities of greater than $800x10^3$ cells/ml were judged to be exhibiting proliferation. Because cultures treated with IL3-Free medium exhibited densities of less than $4x10^3$ cells/ml, these values, represented by the left-most bar for each cell line, are not apparent on the graphs. The "Neu" cell line used in this experiment is the neu/12C cell line. "NT" indicates not tested.

IL-3 Independence Assay

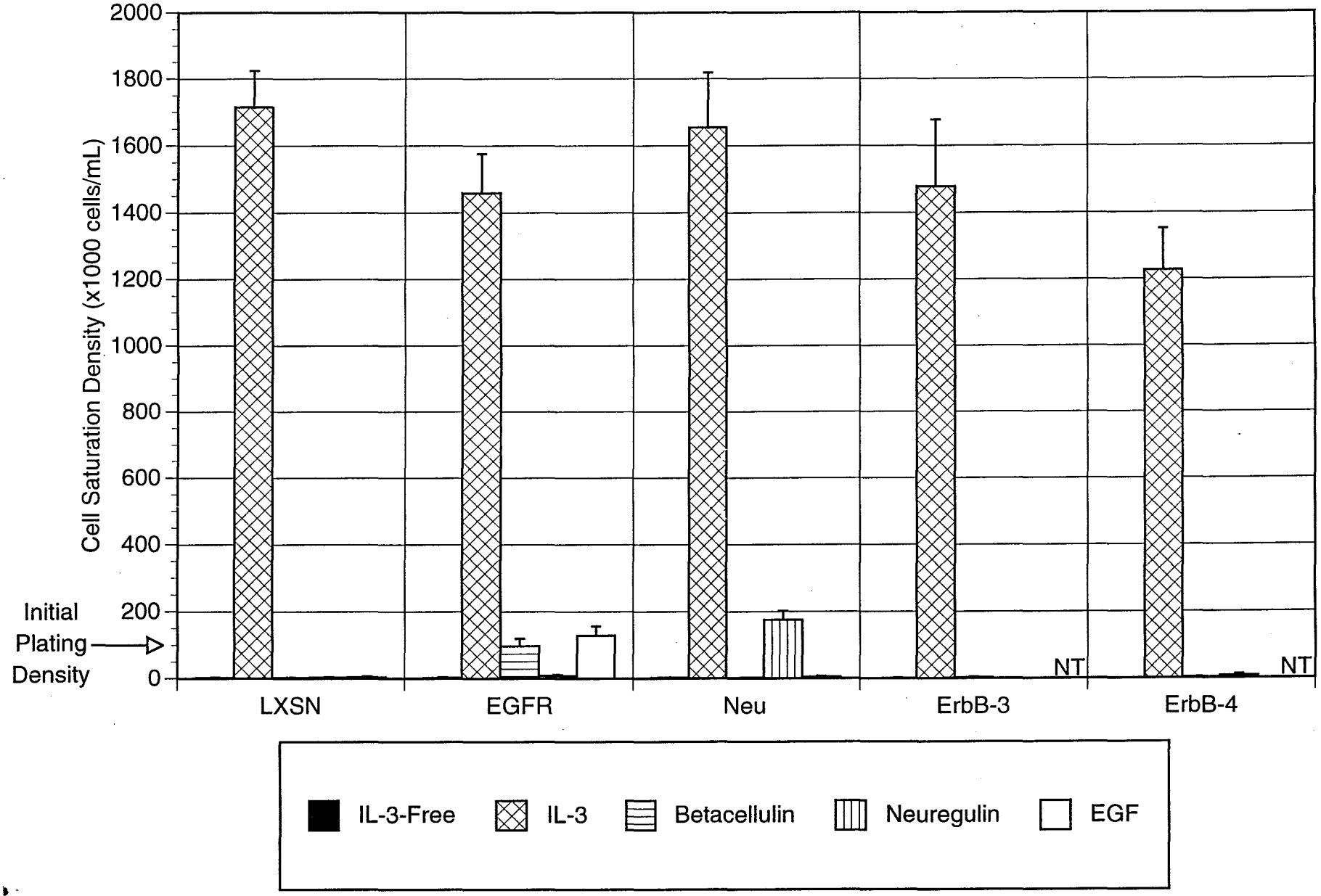

Figure 6a

IL-3 Independence Assay

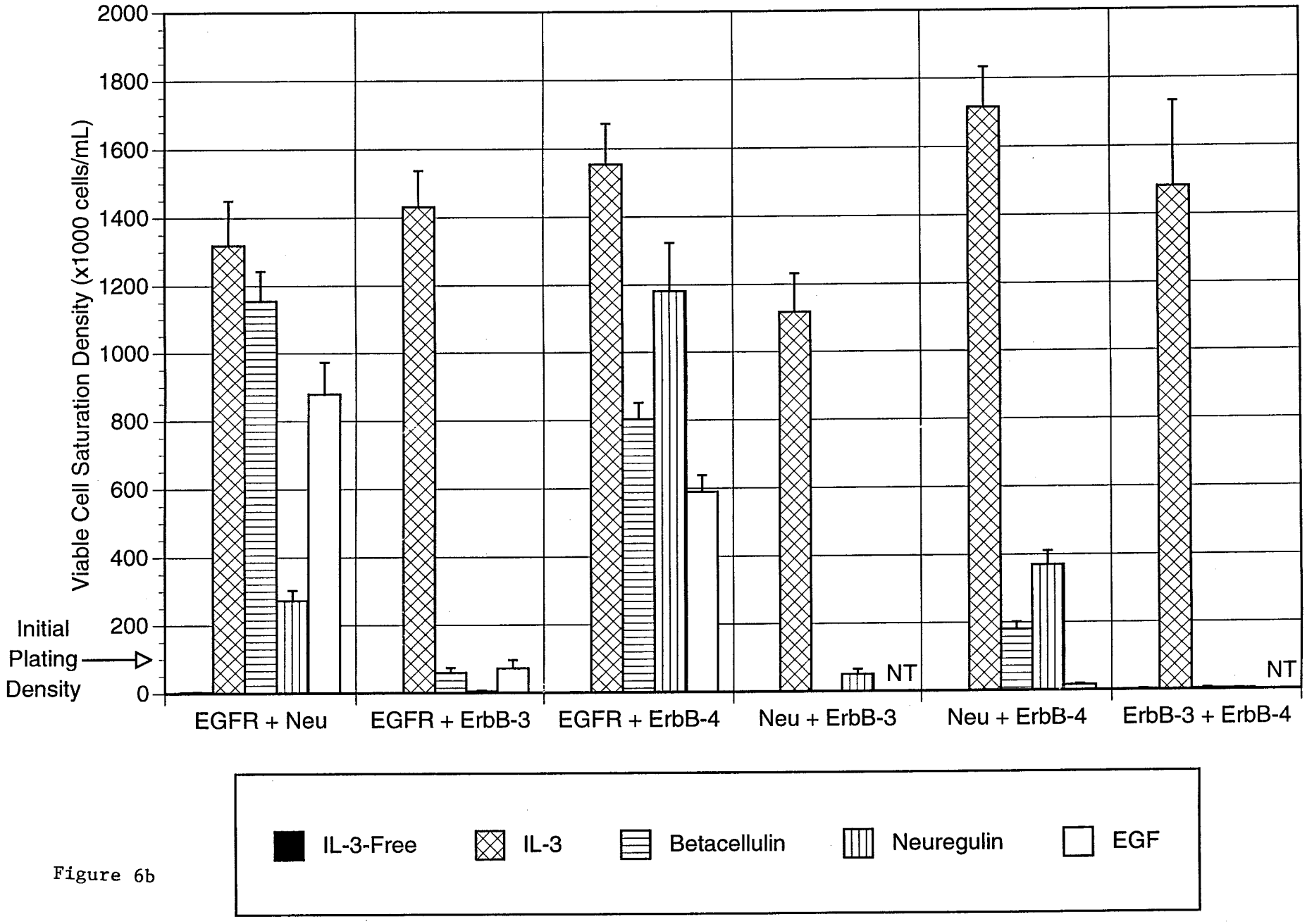

Figure 6b

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Riese DJ II, Bermingham Y, van Raaij TM, Buckley S, Plowman GD, and Stern DF. Betacellulin activates the epidermal growth factor receptor, erbB-4, and induces cellular response patterns distinct from those stimulated by epidermal growth factor or neuregulin-β. *Oncogene*, submitted.

Riese DJ II, van Raaij TM, Plowman GD, Andrews GC, and Stern DF. Cellular response to neuregulins is governed by complex interactions of the erbB receptor family. *Mol. Cell. Biol.*, in press.

Abstracts

Riese DJ II, van Raaij T, Barbacci G, Moyer J, Plowman GD, and Stern DF. Heregulin-induced erbBX signaling. Cold Spring Harbor Meeting on Tyrosine Phosphorylation and Cell Signaling. June, 1995. Cold Spring Harbor, NY.

Riese DJ II, Plowman GD, and Stern DF. Characterization of heregulin-induced signaling. Foundation for Advanced Cancer Studies Tenth Meeting on Oncogenes, June, 1994. Frederick, MD.

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David F. Stern, Ph.D., Supervisor

Graduate degrees resulting from contract support:
None